EFFECT OF CRYOPRESERVATION ON THE INTEGRITY OF COAXIAL ALGINATE CAPSULES

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ABSTRACT

Encapsulation of clinically relevant cells, such as multipotent stromal cells (MSCs), in three-dimensional (3D) core-shell alginate structures is a promising method for the treatment of various diseases as well as drug testing and development. By utilizing the alginate capsules, drugs and active substances can be delivered specifically to the desired place within the patients' body. The aim of this work was to investigate the effect of cryopreservation on the integrity of cell-free coaxial alginate capsules after thawing using slow freezing to ensure their long-term storage. Alginate capsules were produced by electro-spraying and cryopreserved using different cryoprotective agents (CPAs), incubation time and thawing temperature. The results suggest that the capsules loaded with 10% (v/v) dimethyl sulfoxide (DMSO) and 0.3 M sucrose and using high thawing rates demonstrated a beneficial effect on the integrity of capsules, as compared to 10% (v/v) DMSO alone and applying low thawing rates. This study is a further development towards the application of cryopreservation for long-term storage and the emerging cell-based medicine.

Keywords: electro-spraying, tissue cryopreservation, core-shell capsules, alginate hydrogels, membrane

INTRODUCTION

Regenerative medicine includes a wide range of applications, ranging from organ and tissue transplantation to highly sophisticated tissue engineered scaffolds and cell therapeutics [1]. In particular, stem cell-based cell therapies represent a promising and future-oriented method [2]. MSCs are increasingly required for therapeutic applications due to their differentiation ability. In turn, cryopreservation enables a safe and effective long-term storage of cells [2]. This can ensure the sufficient availability of cells [3]. Studies from the Food and Drug Administration (FDA) confirm that 80% of MSCs submissions use the cryopreservation for a long-term storage and preservation of cells [1].

However, freezing MSCs is associated with many complications. Cell encapsulation in hydrogels is a promising alternative to conventional cryopreservation in suspension. Alginate biomaterial is often used for this purpose due to its gel-forming, high biocompatibility and semi-permeable properties. 3D core-shell gel structures can be produced using coaxial electro-spraying, where the cells are centrally positioned inside the alginate capsules. The hydrogel protects the cells from cryodamage and also acts as an extracellular matrix [4]. It also enables the maintenance of cellular metabolic activities. Moreover, the utilization of coaxial alginate capsules has been shown to lower immune responses in patients, circumventing side effects. A variation in membrane thickness and pore size of the envelope membrane allows a better control of diffusion. Consequently, the delivery of cell therapeutics is made controllable [5, 6].

For a successful cryopreservation in the alginate capsules, the osmotic effects during freezing and subsequent thawing, the thermal behavior of the capsules, and the effects of applied CPAs and their incubation time are important [7, 8]. Therefore, the development of a suitable freezing protocol is required. Gryshkov et al. [9, 10] have already produced and optimized the cell-free and cell-laden core-shell capsules. This paper serves to further develop and improve the integrity of cell-free coaxial alginate capsules (Ø 3000 µm) and is performed with a focus on the CPA loading time (45, 90 min), container type (cryovials), CPAs (DMSO, DMSO + sucrose), thawing temperature (37°C, 60°C) and thawing time (60 s). The aim of this parameter study is to obtain the maximum number of intact alginate capsules after cryo-preservation. The capsules were prepared using the

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electro-spraying method. The optimization of parameters is performed with reference to the cell encapsulation of MSCs and is used to improve the cryo-preservation protocol of encapsulated MSCs within hydrogels, consisting of alginate.

RESEARCH CONCEPT

All chemicals used in this study were purchased from Carl Roth (Germany), unless stated otherwise. Low viscosity (molecular weight of 100–200 kDa [11], viscosity of 100–300 cP (2%, 25°C), from Sigma-Aldrich) alginate sodium salt from brown algae was used in the current study.

Preparation of coaxial capsules

The production of cell-free core-shell capsules was conducted using the electro-spraying approach developed by Gryshkov et al. [5, 9]. The general process of electro-spraying included the pumping of the alginate solution and cell suspension at defined flow rates through a coaxial nozzle, the application of a high voltage between the nozzle and gelling solution and the transportation of the produced alginate droplets to a bath containing a gelling solution of 100 mM calcium chloride ($CaCl_2$, Carl Roth) for cross-linking. For the core-shell capsules sodium alginates were dissolved in 10 mM 4-(2-hydroxyethyl)-1-piperazi-neethanesulfonic acid (HEPES, pH 7.4) prepared in 0.9% sodium chloride. The final concentration of alginate solution was 2.5% (w/v). The membrane (alginate) and core (HEPES) solutions were pumped simultaneously at different flow rates through the custom developed coaxial nozzle. After gelling for 30 min, the alginate capsules were collected and washed once with a washing solution (20 mM $CaCl_2$ in 10 mM HEPES containing 0.9% NaCl (pH 7.4) and used for further analysis.

The alginate and HEPES solutions were pumped through an outer (outer diameter 1.83 mm, B Braun) and inner (outer diameter 0.4 mm, B Braun) needle using two syringe pumps (KD Scientific). The coaxial nozzle was charged positively, whereas an electrode was grounded and immersed into the gelling solution. The distance between the tip of the outer needle and the surface of the gelling solution represents the spraying distance. The following parameters were applied to produce the alginate capsules with a centrally located core and with an outer diameter of 3000 ± 99 µm, an inner diameter of 1700 ± 90 µm and a membrane thickness of around 650 µm: a high voltage of 12 kV and a ratio of the flow rate of the alginate to the inner fluid (HEPES) of 4:20 (ml/h : ml/h).

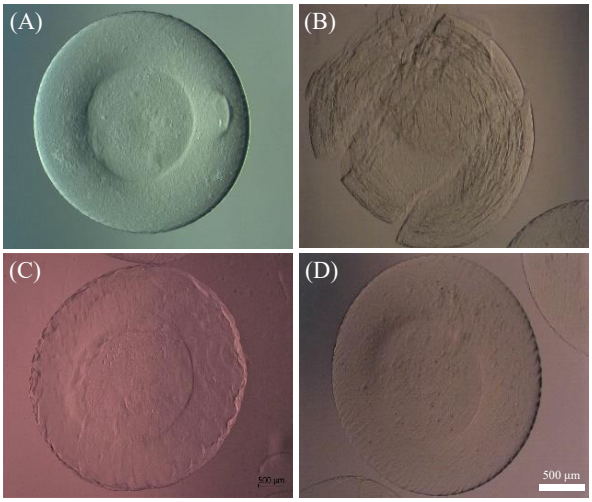

Figure 1: Prepared capsules before freezing (**A**) and the damaging effects of the capsules after thawing (**B–D**). Destroyed capsules (**B**). Damaged capsules (**C**). Here the membrane is not intact and the gel structure is severely damaged. Intact capsules that have an intact outer layer, minor membrane damage, a barely visible core, and a highly visible core (**D**). Scale bars are 500 µm.

Cryopreservation

After washing, the prepared alginate capsules were transferred into cryovials (TPP) with 20 capsules per vial and placed on ice for CPA loading. Afterwards they were incubated for 45 and 90 min in two different cryoprotective solutions: 10% DMSO (DM) and 10% DMSO with 0.3 M sucrose (Sigma-Aldrich) (DMS). The cryoprotective solutions were prepared in a pure cell culture medium supplemented with Fetal Bovine Serum (FBS, Biochrom) in a final concentration of 20% (v/v). In this study, the capsules were frozen "in air", which included removal of the excess of the CPAs after loading. The samples were frozen with 1 K min^{-1} to -80°C in the controlled rate freezer Planer Kryo 560-16 (Planer) and stored in liquid nitrogen in a cryogenic (Fischer Scientific) at -140°C for at least 7 days. Thawing was realized in a water bath pre-warmed to 37°C or 60°C with gentle shaking for 60 s. The last thawing step included addition of pre-warmed Dulbecco's modified Eagle's (DMEM, Biochrom) (37°C or 60°C). After thawing, the capsules were transferred into 6-well culture plates (TPP) and used for further analysis. Analysis and imaging of the capsules were performed using an AxioVert. A1 microscope and ZEN Blue software (Zeiss). The data referring to the capsule integrity was derived according to the damaging effects to the alginate capsules as shown in Figure 1: Category B (destroyed), Category C (damaged) and Category D (intact).

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Statistical Analysis

Each experiment was repeated three times. The results are represented as mean ± standard deviation (SD) and the values of the intact capsules were assessed for normality according to the applied Shapiro-Wilk test.

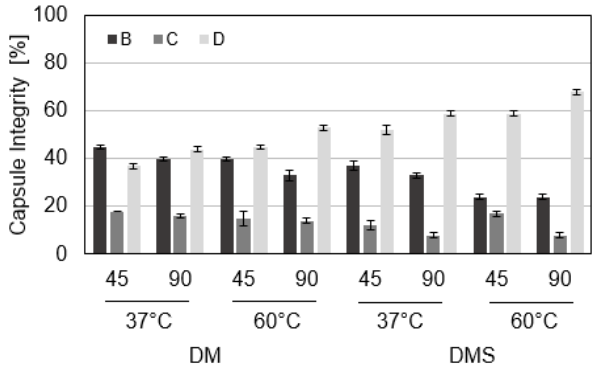

Figure 2: Capsules' integrity after thawing with an incubation time of 45 and 90 min in 10% DMSO (DM) and 10% DMSO with 0.3 M sucrose (DMS). Categorized as [B] destroyed, [C] damaged, and [D] intact. (n = 120).

RESULTS

In this study, core-shell capsules were produced from freshly prepared alginate solutions and samples were analyzed before and after freezing. The effect of 10% (v/v) DMSO (DM) for the applied incubation time of 45 and 90 min (Figure 2) showed that the number of intact alginate capsules increased with an increasing thawing temperature and incubation time. For an incubation time of 90 min and a thawing temperature of 60°C, 45% of alginate capsules were intact. The percentage for (C) was 15% and for (B) 40%. The lowest capsule integrity was obtained with the incubation time of 45 min and the temperature of 37°C. The percentage of intact capsules was 28%. Completely destroyed were 60% and damaged 13%.

Applying the second cryoprotective solution, DMS had different effects on the capsule integrity (Figure 2). The result show that the application of sucrose led to a decrease of destroyed and damaged capsules. Consequently, the samples that were thawed at 37°C and incubated for 45 min had a percentage of 52% of intact capsules. Therefore, these were classified in (D). The percentage of capsules assigned to (C) was 12% and for (B) the percentage was 37%. Increasing the temperature to 60°C, while keeping the incubation time constant at 45 min, caused an increase in intact capsules. The percentage of capsules allocated to (D) was 59%. 17% of the capsules were considered damaged and 24% were completely destroyed. The results (Figure 2) show the effect of the incubation time of 90 min on the capsule integrity. Applying a thawing temperature of 60°C and an incubation time of 90 min accounted for 68% of intact capsules. The best overall results were obtained by using this combination of parameters. Here, 24% of the capsules were found completely destroyed and 8% damaged.

DISCUSSION

Alginate has been investigated and used therapeutically and diagnostically for many biomedical applications due to its biocompatibility, low toxicity and relatively low cost [6, 12]. The semi-permeability of the alginate hydrogel allows the transport of drugs, nutrients, oxygen as well as metabolic products through the alginate, but prevents the attack of high molecular weight substances such as antibodies on the cells transported within the alginate capsules. The idea behind using electro-spraying approach is a coaxial flow of two liquids: alginate as an outer membrane and a cell suspension as a core. Moreover, the core-shell capsules size and the thickness of the alginate membrane can be varied [9, 10]. Based on the results obtained, it can be seen that the alginate hydrogel is an important factor in successfully preserving the alginate capsules using the optimal freezing protocols. Cryopreservation of the low-viscosity alginate capsules was successfully carried out with different percentages of CPAs, independent of the incubation time and thawing temperature.

In the current study several damaging effects can be seen which occur during cryopreservation. This could be due to several reasons: short incubation time of CPAs, poor penetration of CPAs into the alginate capsules, without using of sucrose as well as the thawing time and temperature.

Herein, we also analyzed the effect of the application of sucrose and high thawing temperatures. The utilization of a longer incubation time allowed an extended penetration of the CPA into the alginate capsules. Due to this, the protective properties increased which was reflected in the increase of intact capsules [10]. The longer incubation time caused a 6% increase in capsule integrity. The use of sucrose also supported the capsule integrity due to the fact that sucrose promoted dehydration and reduced the water content in the capsules [13]. Consequently, it prevented capsule bursting, and reducing the number of destroyed capsules. The addition of sucrose resulted in an average increase of 15% in integrity. Although a better result was obtained by the longer incubation time, it is inadvisable

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to increase the penetration time of CPAs. In case a colonization of cells occurs, CPAs may be toxic to the cells over a longer period of time [2, 14].

CONCLUSIONS & OUTLOCK

In this paper we showed the effect of cryopreservation on the integrity of cell-free coaxial alginate capsules. The study was carried out for different process parameters: variation of the incubation time, CPAs and thawing rates. The aim of this study was to analyze the combination of parameters that showed the maximum number of intact alginate capsules and was performed in view of establishing the freezing protocol for encapsulated MSCs. In this study, we observed that the percentage of intact capsules increased with increasing incubation time and thawing temperature. The addition of 0.3 M sucrose to 10% DMSO caused an increase in capsule integrity. Almost 70% of the capsules survived after thawing and short-term storage. For the further optimization of the freezing protocol, which could result in an increase of intact alginate capsules, would be interesting: increasing the sucrose content, analysis of different cooling rates, optimization of freezing by applying cryobags as well using electroporation-assisted delivery of sucrose and trehalose or similar into MSCs.

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